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Precocious appearance of markers of squamous differentiation in metaplastic cells of human endocervix

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Summary. We used immunoperoxidase methods employing anitbodies against involucrin and filaggrin, both markers of squamous terminal differentiation, to study squamous metaplastic transformation in the human endocervix. Expression of involucrin and filaggrin was restricted to squamous metaplastic cells whereas columnar epithelial cells were constantly negative. Immature squamous metaplastic epithelium also showed a positive immunostaining. In mature squamous metaplasia a suprabasal homogeneous staining pattern similar to that found in the exocervical epithelium was detected, although with full-thickness filaggrin immunoreactivity in 45% of cases ($P < 0.05$). These results support the hypothesis of an epithelial origin of reserve subcolumnar cells, and suggest that precocious squamous differentiation seems to take place in metaplastic cells of the human endocervix.

Key words: Filaggrin – Involucrin – Squamous metaplasia – Uterine cervix

Introduction

The physiological process by which the endocervical columnar epithelium becomes replaced by squamous metaplastic epithelium in the transformation zone (TZ) is poorly understood. The histogenesis of the TZ remains controversial. It has been established that squamous metaplastic epithelium is formed by proliferation and gradual transformation of the so-called reserve subcolumnar cells [3, 26], which appear to have the ability to become differentiated into either columnar cells or squamous cells under the influence of certain local environmental and hormonal factors [1, 30]. The TZ is considered to be the main site of origin of all premalignant and malignant lesions of the uterine cervix [2, 3, 15, 27].

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The availability of specific markers of epithelial squamous terminal differentiation offers the possibility of studying dynamically the gradual transition from columnar to squamous cervical epithelium, via immature and mature squamous metaplasias.

Involucrin is a 140 KD cytoplasmic protein [37], synthesized exclusively in maturing cells of human stratified squamous epthelium, which represents the major precursor of the cross-linked envelope formed immediately beneath the cellular plasma membrane of superficial squamous epithelial cells [5, 25, 32].

Filaggrin is a 37 KD histidine-rich protein [12, 16, 23], which acts as a protein matrix that induces the aggregation of keratin filaments in the upper cell layers of squamous epithelia [7, 14].

In the epidermis, involucrin and filaggrin are usually not found in basal and suprabasal cell layers, and are first detected in the upper spinous layer [19, 20, 25, 31, 37]. Accordingly, both markers do not appear before the onset of terminal differentiation and can be therefore regarded as specific markers of normal squamous differentiation and maturation [4, 8, 18, 21, 37].

Although previous immunohistochemical studies of involucrin and filaggrin have been carried out in a variety of normal and neoplastic cervical tissues [4, 9, 11, 38], no studies focusing on endocervical squamous metaplastic epithelia have apparently been done.

The aim of the present study was to analyze by immunohistochemical methods involucrin and filaggrin expression in squamous metaplastic cells of the human endocervix in order to improve our knowledge about the squamous metaplastia.

Materials and methods

A total of 40 selected colposcopically directed cervical punch biopsies of the TZ diagnosed by histologic criteria [2] as endocervical squamous metaplasia and graded as immature squamous metaplasia (24 cases) or mature squamous metaplasia (16 cases), were included in our study. In addition, sections of original exocervical (10 cases) and endocervical (10 cases) mucosa were also studied for purposes of comparison. The aforementioned material was obtained from patients referred to the Department of Gynecology at the Valencia University School of Medicine. The mean age of the patients was 32 years (range 21–44) and all were sexually active. All diagnoses were confirmed by at least two pathologists. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and processed by the usual method for hematoxylin-and-eosin-staining.

For the immunohistochemical study, paraffin sections 3 μ-thick were cut, allowed to dry overnight at 56°C, deparaffinized in xylene and toluene, rehydrated in alcohols and incubated in 0.3% hydrogen peroxide (20 min) for blocking endogenous peroxidase activity.

For involucrin immunostaining, an indirect immunoperoxidase technique was used [17]. Briefly, after incubation in normal goat serum (20 min) to reduce non-specific labelling, sections were incubated sequentially with a polyclonal rabbit anti-human involucrin antibody (Biomedical Technologies Inc, BT-600, Stoughton, USA) (45 min) and a goat anti-rabbit IgG-horseradish peroxidase (35 min).

For filaggrin immunostaining, an avidin-biotin-peroxidase technique was employed using a commercially available immunokit (Vectastain ABC kit, PK4002, Vector, Burlingame, USA). After blocking non-specific staining with normal horse serum (20 min), the sections were incubated sequentially with a monoclonal anti-human filaggrin antibody (Biomedical Technologies Inc, BT-576, Stoughton, USA) (60 min), a biotinylated anti-mouse IgG (45 min) and an avidin-biotin-peroxidase complex (60 min).

Between steps, all sections w BioMérieux, France). Both im ning 8 mg of 3-amino-9-ethylca (Riedel de Haën, Hannover, F 5.2). The sections were counte containing gelatin (20 g) in gl included in each reaction. Sectio only suprabasal immunoreacti obtained by omitting the prima

The staining pattern for in Intensity of staining (negative, w full-thickness), epithelial distrib cytoplasmic versus "peripheral"

For statistical comparisons Chi-square analysis with Fisher's as statistically significant.

Results

Exocervical squamous e involucrin and filaggrin. increasing intensity towar cases (Fig. 1). Basal cells negative for both markers

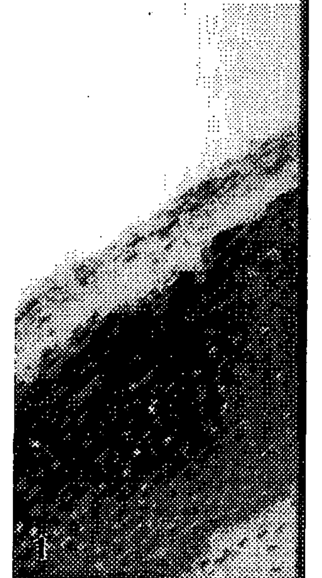

Fig. 1. Paraffin section of squa antibody showing a suprabasal h reactive and unreactive cells. (ethylcarbazole and counterstaine

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Between steps, all sections were rinsed with phosphate-buffered saline solution at pH 7.4 (PBS, BioMérieux, France). Both immunoperoxidase reactions were demonstrated by a solution containing 8 mg of 3-amino-9-ethylcarbazol (Sigma, St. Louis, USA) in 1 ml of N-N-dimethylformamide (Riedel de Haën, Hannover, FRG), 0.2 ml of hydrogen peroxide and 20 ml of acetate-buffer (pH 5.2). The sections were counterstained with Mayer's haematoxylin and mounted with a medium containing gelatin (20 g) in glycerin (175 ml) and distilled water (150 ml). Control slides were included in each reaction. Sections of normal skin were employed as a positive control and revealed only suprabasal immunoreactivity with no background staining. Negative control slides were obtained by omitting the primary antibody and were consistently negative.

The staining pattern for involucrin and filaggrin was evaluated by two different observers. Intensity of staining (negative, weak positivity or strong positivity), extent of staining (suprabasal or full-thickness), epithelial distribution (homogeneous or irregular) and cellular localization (diffuse cytoplasmic versus "peripheral" cytoplasmic) were recorded in each case.

For statistical comparisons between the native and metaplastic squamous epithelial tissues, a Chi-square analysis with Fisher's correction was performed. A P value less than 0.05 was considered as statistically significant.

Results

Exocervical squamous epithelium exhibited similar patterns of staining for involucrin and filaggrin. A strong suprabasal uniform staining pattern with increasing intensity towards the upper layers of the epithelium was present in all cases (Fig. 1). Basal cells and immediately adjacent parabasal cells were entirely negative for both markers. Therefore, an abrupt boundary between reactive and

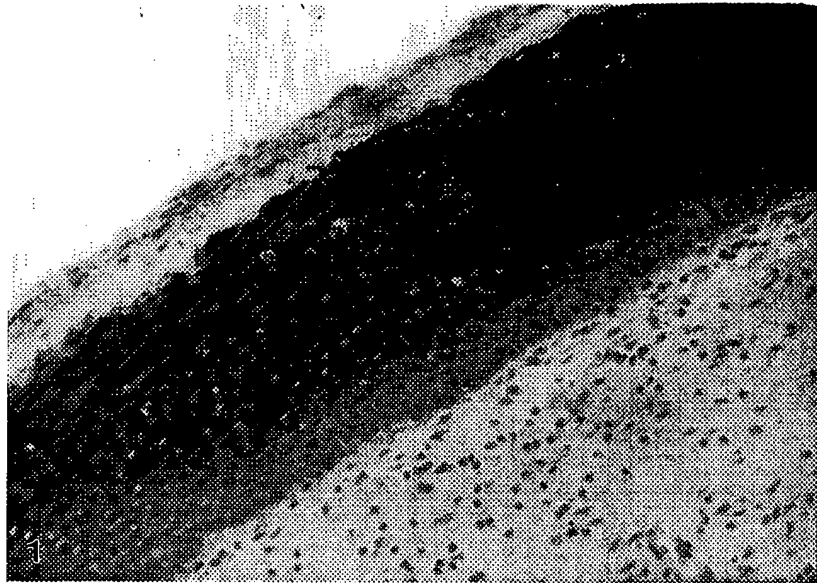

Fig. 1. Paraffin section of squamous cervical epithelium labelled with anti-involucrin polyclonal antibody showing a suprabasal homogeneous staining pattern. Note the abrupt boundary between reactive and unreactive cells. (Indirect immunoperoxidase technique developed with 3-amino-9-ethylcarbazole and counterstained with Mayer's hematoxylin. × 182)

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unreactive cells was detected in the majority of cases. Within each cell, both proteins were diffusely distributed throughout the cytoplasm, but frequently a reinforcement of involucrin immunostaining was evident at the periphery of the cell, just below the cellular plasma membrane.

By contrast, involucrin and filaggrin were completely absent from the original columnar endocervical epithelium (Fig. 2), except in an area of initial squamous metaplasia, unnoticed histologically, where both proteins were limited to the reserve subcolumnar cells with apparent squamous metaplastic transformation (Fig. 3).

In sections of metaplastic squamous epithelial tissues filaggrin expression closely matched involucrin distribution. Hence, columnar endocervical cells were consistently negative while squamous metaplastic cells always showed a diffuse and intense immunoreactivity for involucrin and filaggrin, even in the most immature squamous metaplastic epithelium (Fig. 4). Instead, a diffuse homogeneous staining pattern very similar to that found in the native exocervical stratified squamous epithelium was observed in sections of mature squamous metaplasia (Fig. 5, 6), although weaker and with a less evident boundary between reactive and unreactive cells. In fact, as contrasted to the original exocervix filaggrin was distributed throughout the whole thickness of the mature squamous metaplastic epithelium in 45% of the cases ($P < 0.05$), while involucrin showed a full-thickness immunostaining in 10% of the mature metaplastic transformations studied (P = not significant).

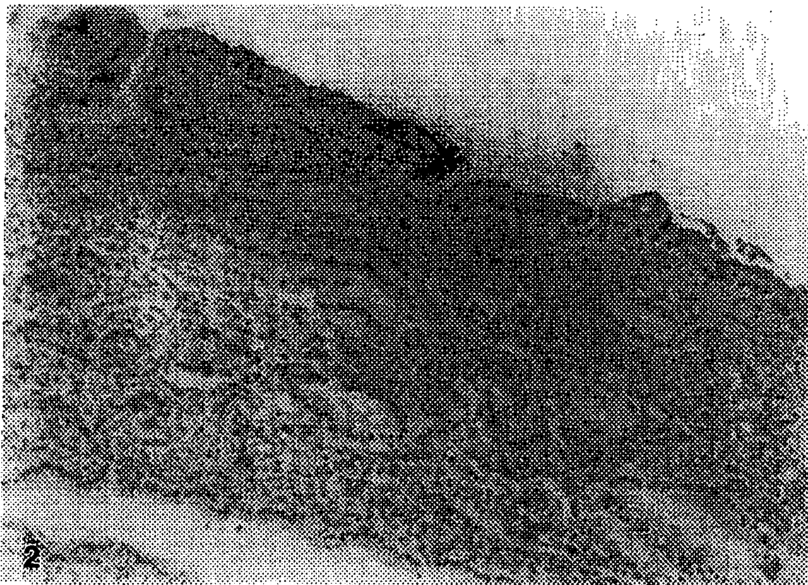

Fig. 2. Complete absence of filaggrin in columnar endocervical epithelium. (Avidin-biotin complex immunoperoxidase technique demonstrated with 3-amino-9-ethylcarbazole and counterstained with Mayer's hematoxylin. × 91)

Fig. 3. Section of normal end
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Fig. 4. Endocervical immature
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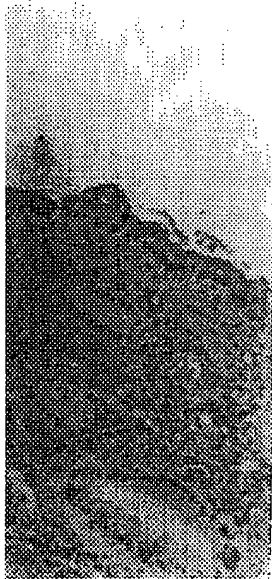

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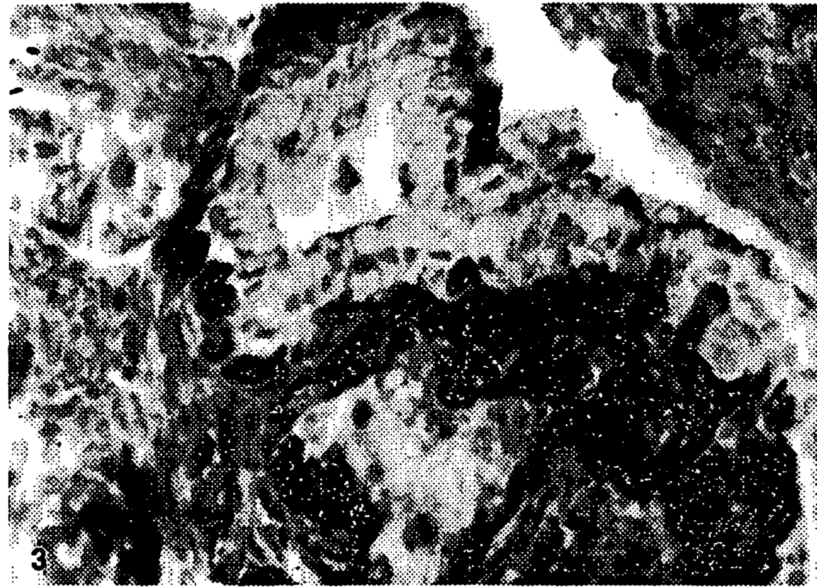

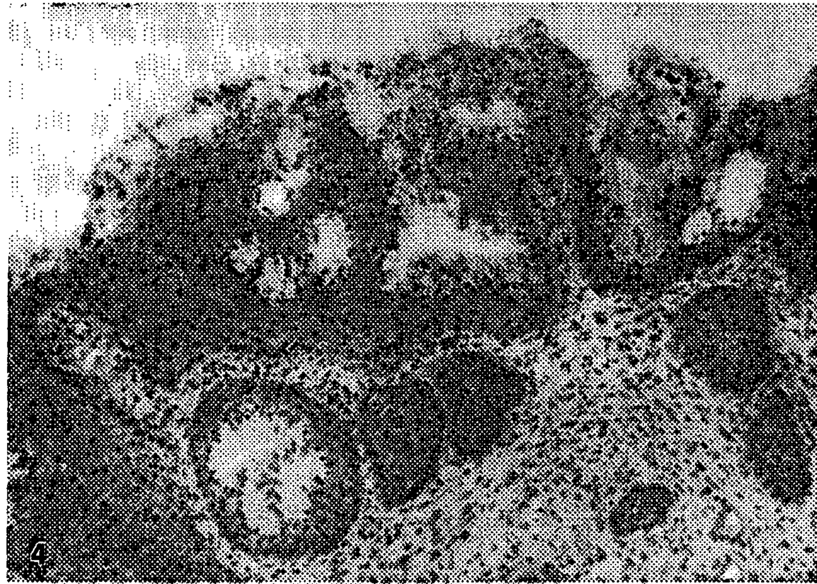

Fig. 3. Section of normal endocervix labelled with anti-involucrin polyclonal antibody showing immunoreactivity restricted to the reserve subcolumnar cells with apparent squamous metaplastic transformation (black) and lack of involucrin in columnar epithelial cells. (Indirect immunoperoxidase technique performed using 3-amino-9-ethylcarbozole and counterstained with Mayer's hematoxylin. × 364)

Fig. 4. Endocervical immature squamous metaplasia with diffuse immunostaining for filaggrin in metaplasic elements. Note the absence of filaggrin in columnar epithelial cells. (Avidin-biotin complex immunoperoxidase technique developed with 3-amino-9-ethylcarbazole and counterstained with Mayer's hematoxylin. × 91)

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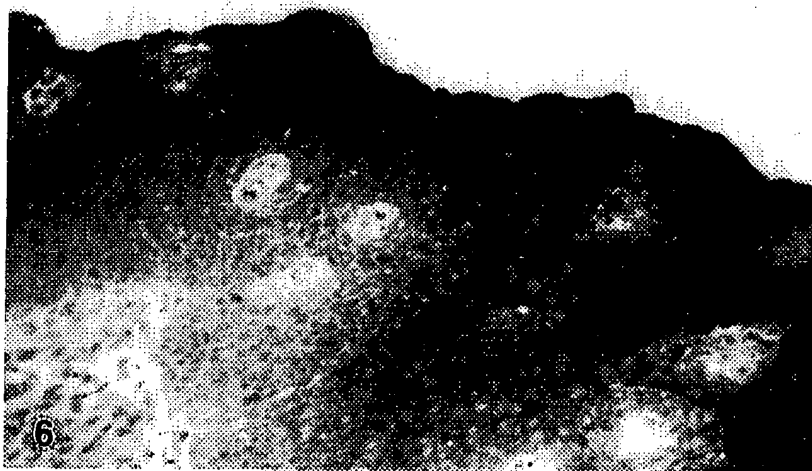

Discussion

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Discussion

This preliminary study attempts to show the expression of two markers of squamous differentiation in endocervical squamous metaplastic epithelia. Both involucrin and filaggrin appeared to behave as highly sensitive and specific markers of squamous metaplasia in tissue from the TZ of the uterine cervix. Similar findings were obtained with both markers.

Previous studies have demonstrated the presence of involucrin in metaplastic squamous epithelium of amnion, umbilical cord, urinary bladder, lung, endocervix and endometrium [28, 29, 34–36]. In all such conditions involucrin immunoreactivity was limited exclusively to squamous elements.

In our series, a surprising finding was evident in cases of endocervical immature squamous metaplasia, in which very incipient squamous metaplastic cells were able to synthesize markers of terminal squamous differentiation, such as involucrin and filaggrin. The importance of these observations remains to be determined, but strongly suggests that terminal differentiation sequence seems to be somehow accelerated in these conditions.

Attending to classical histologic criteria [13], the process of squamous metaplasia in human endocervix has been divided by Fluhmann in five stages of development. Squamous metaplasia can be first demonstrated "histologically" by the appearance of a single row of reserve subcolumnar cells, undifferentiated at this moment, immediately beneath the columnar endocervical epithelial cells (Fluhmann's stage I). The number of undifferentiated reserve cells increases gradually from 3–5 cell layers in Fluhmann's stage II to 6–10 cell layers in Fluhmann's stage III. According to this author, squamous differentiation occurs initially in the upper layers of the metaplastic epithelium at stage III. Later on, squamous maturation and differentiation is completed in the inner layers of the metaplastic epithelium (Fluhmann's stage IV), until a totally developed stratified squamous epithelium similar to the native exocervical epithelium is formed (Fluhmann's stage V).

The precocious appearance of involucrin and filaggrin in early squamous metaplastic epithelia (histologic stages I, II) seriously questions the metaplastic sequence of development reported by Fluhmann [13]. Apparently, reserve subcolumnar cells are able to synthesize products of mature squamous epithelial cells as soon as they become differentiated into squamous metaplastic cells. In our opinion, the finding of reserve subcolumnar cells with positive reaction for involucrin and filaggrin rules out their supposed mesenchymal origin, as juzged

Fig. 5. Focal suprabasal staining of involucrin in mature squamous metaplastic epithelium. This suprabasal immunostaining pattern closely resembles involucrin distribution found in the normal exocervix. (Indirect immunoperoxidase technique demonstrated with 3-amino-9-ethylcarbazole and counterstained with Mayer's hematoxylin. × 182)

Fig. 6. Mature squamous metaplastic cervical epithelium showing a diffuse suprabasal homogeneous staining pattern of filaggrin similar to that found in original squamous cervical epithelium. (Avidin-biotin complex immunoperoxidase technique performed using 3-amino-9-ethylcarbazole and counterstained with Mayer's hematoxylin. × 91)

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by the absence of these antigens in stromal cells [6, 8]. Puts et al. [26] arrived at the same conclusion based on the presence of keratins and the absence of vimentin (an intermediate filament protein characteristic of non-epithelial cells) in these reserve subcolumnar cells.

Once the squamous metaplastic process is finished, terminal differentiation closely resembles that found in the original exocervical epithelium, as juzged by the suprabasal homogeneous staining pattern of involucrin and filaggrin obtained in mature squamous metaplastic epithelial sections.

However, immunohistochemical characterization of the TZ is still controversial. Previous reports have analyzed the distribution of other markers in squamous metaplastic cells of human endocervix. The proposed bipotential nature of subcolumnar reserve cells [1] may explain the finding of immature metaplastic cells coexpressing cytokeratins typical for glandular epithelium next to cytokeratins typical for keratinizing squamous epithelial tissues [26]. But differentiation of reserve cells into nondividing endocervical columnar cells is known to be followed by a concomitant loss of cellular keratin proteins by a mechanism not yet understood [30].

On the other hand, it seems evident that reserve subcolumnar cells share some antigenic determinants with cells from the basal layer of normal exocervix because both types of cells can be detected using antibodies specifically directed against prekeratins [1], human transferrin receptor [24], the tissue polypeptide antigen [22, 33] or the 24K protein [10].

In conclusion, a precocious squamous differentiation seems to take place in metaplastic cells of human endocervix but more work is needed to elucidate the precise sequence of events and the molecular mechanisms by which they occur.

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